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Effects of Long-Term Treatment with Acetylene on Nitrogen-Fixing Microorganisms

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Long periods of experimental incubation with acetylene led to a multifold enhancement of acetylene-reducing activity in *Anabaena cylindrica, Anabaenopsis circularis, Rhodospirillum rubrum,* and *Azotobacter vinelandii.* Rates of acetylene reduction showed a gradual increase and reached a peak after 2 to 6 h of continuous incubation under acetylene. Thereafter, enzyme activity rapidly declined. A similar enhancement of ethylene production was observed when pretreatment with acetylene was interrupted periodically by a brief exposure to ambient (or oxygen-free) atmosphere without acetylene although the decline of acetylene-reducing activity was less rapid. Pretreatment with acetylene depressed photosynthetic $^{14}CO_2$ fixation and $^{15}N_2$ incorporation in *Anabaena cylindrica.* It is concluded that assessments based on long-term experimental incubation with acetylene may grossly overestimate the actual quantities of fixed nitrogen in the field.

Nitrogenase reduces a variety of substrates, like azide, nitrous oxide, acetylene, cyanides, and isocyanides, most of which are structural analogs of dinitrogen. The discovery of the nitrogenase-catalyzed reduction of acetylene to ethylene (6; R. Schöllhorn and R. H. Burris, Fed. Proc. **25**:710, 1966) and the application of this reaction as a sensitive, rapid, and simple assay to measure nitrogen fixation (11, 17) has contributed greatly in recent years to progress in research on biological nitrogen fixation. The biochemical basis of the assay and its application to laboratory and field measurements of nitrogenase activity have been examined and reviewed in detail (9, 10, 14).

In certain field situations in which the numbers of potential nitrogen-fixing organisms are small or the conditions of nitrogen fixation are not favorable, the temptation may arise to apply long periods of experimental incubation with acetylene to compensate for the low rates of acetylene reduction. Although such a practice is generally considered undesirable (9), little is known about the effects of long-term treatment with acetylene.

In this study we investigated the effect of preincubation under acetylene upon a variety of nitrogen-fixing organisms, including *Anabaena cylindrica, Anabaenopsis circularis, Azotobacter vinelandii,* and *Rhodospirillum rubrum.*

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MATERIALS AND METHODS

Organisms and growth conditions. Batch cultures of *Anabaena cylindrica* (Cambridge Culture Collection no. 1403/2a) were grown axenically in a medium free from combined nitrogen (1), or in a similar medium containing 1.0 mM potassium nitrate or 1.0 mM diammonium hydrogen phosphate, in air at 25 ± 2°C, shaken continuously at 90 oscillations/min, and illuminated with white fluorescent light of 3,500-lx intensity.

Anabaenopsis circularis (Cambridge Culture Collection no. 1402/1) was grown in a similar medium under ambient atmosphere in a continuous-culture apparatus (8) at 25°C and 1,200-lx light intensity, aerated, and stirred magnetically.

Azotobacter vinelandii (National Collection of Industrial Bacteria, Torry Research Station no. 8789) was cultivated in Burk nitrogen-free medium as modified by Strandberg and Wilson (18) at 30°C under air on a reciprocal shaker set at 120 reversions/min.

R. rubrum (National Collection of Industrial Bacteria, Torry Research Station no. 8255) was grown anaerobically under a gas phase of 1% CO_2 in N_2, in a medium described by Ormerod et al. (13) but free from ammonium sulfate, at 30°C and illuminated with tungsten light of 3,000-lx intensity.

Assay methods. Total nitrogen content of cell material was determined by a standard Nessler procedure after Kjeldahl digestion and distillation.

^{15}N incorporation was assayed according to the method of Burris and Wilson (3), as modified by Ross and Martin (15), using an Associated Electrical Industries MS2 mass spectrometer.

Nitrogenase activity was measured by the acetylene-ethylene assay (17). Acetylene was provided at a partial pressure of 0.1 atm, except where otherwise

indicated. Three-milliliter cell suspensions in calibrated serum bottle-type reaction vessels of about 7.5-ml capacity were continuously agitated on a rotor at 32 rpm for 30 min. At the end of the assay period, part of the gas phase in the reaction vessel was transferred into a sealed pre-evacuated tube, and triplicate gas samples were analyzed for ethylene in a Varian Aerograph 1200 gas chromatograph fitted with a Porapak R column and a hydrogen flame ionization detector.

Acetylene was determined by means of reaction with alkaline potassium mercuric iodide, followed by quantitative (turbidometric) determination of the precipitate (mercuric acetylide) formed (4).

For radioactivity assay, samples of cell material incubated with ^{14}C-labeled sodium bicarbonate were filtered on membrane filters (0.45-μm pore size; Millipore Corp.), prewashed with cold bicarbonate, and washed free of tracer before drying at 110°C for 15 min and transferring into KL372 liquid scintillator (Koch-Light Laboratories Ltd.) for counting in a Nuclear Enterprise Ltd. type 6500 liquid scintillation counter.

Monochromatic light was provided by a Bausch and Lomb 500-nm grating monochromator. Radiation energy was measured with a Kipp and Zonen CAI-type compensated thermopile, exposing a diaphragm area of 1 cm^2, connected to an AL4 Microva-type microammeter.

Experimental treatment. Three parallel basic treatments were applied during these studies. In treatment A, replicates of the cell suspension were incubated for several hours under 10% acetylene in air (vol/vol), and ethylene production was determined on triplicate samples at hourly intervals. Treatment B consisted of alternate exposure of the cell suspension to a gas phase of 10% acetylene in air and ambient atmosphere, respectively, each period lasting for 30 min; this was partly to avoid long periods of nitrogen starvation due to the inhibition of N_2 fixation by acetylene, and partly to prevent undesirable changes in the composition of the gas phase. Again, triplicate samples were taken at hourly intervals for acetylene-ethylene assay. In control treatment C, all samples were incubated under ambient atmosphere without acetylene, and triplicate samples were assayed at hourly intervals. All reaction vessels were continuously agitated at 90 oscillations/min to enhance gaseous exchange.

RESULTS

Solubility of acetylene in water. Acetylene solubility was tested in relation to the experimental treatments used in distilled water, culture medium, and culture filtrate. The solubility of acetylene in water (1 volume of C_2H_2 in 1 volume of water under 1.0 atm and at 20°C) is about 60 times higher than that of gaseous nitrogen and 8.45 times more than that of ethylene. The solubility is not affected appreciably by the inorganic nutrients supplied in the culture media used or by the accumulation in the growth medium of organic extracellular products.

When water (or aqueous solution) is exposed in a closed vessel to a gas phase containing 0.1 atm of acetylene in air (10%, vol/vol) for 60 min with continuous agitation, the amount of acetylene in solution will reach 15.6% saturation (Table 1). Incubation for only 30 min decreases this value to 11.3% saturation. When incubation under 0.1 atm of acetylene for 30 min is followed by exposure of the water samples to ambient atmosphere for another 30 min, about 68% of the acetylene in solution is lost, 32% still remaining in solution. When free diffusion is supplemented with a subsequent treatment of evacuation (using a rotary pump at 10^{-1} mm of Hg) and flushing with N_2 gas for 10 min, the residual acetylene is reduced to 4.8% of the original amount in solution. This corresponds to a partial pressure of acetylene of about 0.005 atm, equivalent to the K_m (Michaelis constant) for nitrogenase in whole cells (9).

Acetylene dissolved in water can be removed more effectively by bubbling with N_2 gas (Fig. 1). Almost 85% of the dissolved acetylene is displaced by bubbling with N_2 (at a rate of 48 ml through 3 ml of liquid per min) for 1 min, and no traces of acetylene remain in water after bubbling for a total of 4 min.

Effect of preincubation with acetylene on the rate of acetylene reduction by blue-green algae. When *Anabaena cylindrica* was exposed at 20°C and 1,500 lx to 10% acetylene in air with no interruption (treatment A), ethylene production followed a sigmoidal course (Fig. 2a). The rate of acetylene reduction increased exponentially during the first 3 h and declined thereafter, as indicated by the logarithmic plot of nitrogenase activity in Fig. 2b. Maximum rates of ethylene production were measured in samples subjected to 3 h of uninter-

TABLE 1. *Amounts of acetylene dissolved in water after treatments applied in this study*[a]

Treatment	Dissolved C_2H_2 (mg/liter of water)	Saturation (%)
60-min incubation under 0.1 atm of C_2H_2	181.0	15.6
30-min incubation under 0.1 atm of C_2H_2	130.7	11.3
30-min incubation under 0.1 atm of C_2H_2, followed by 30-min incubation under ambient atmosphere	41.7	3.6
30-min incubation under 0.1 atm of C_2H_2, followed by 30-min incubation under ambient atmosphere and 10-min evacuation and flushing (3×) with N_2	6.3	0.5

[a] All incubations were at 20°C and with continuous agitation. Percentage saturation = (C_2H_2 concentration × 100)/solubility.

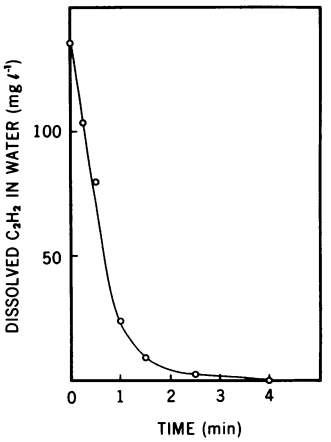

FIG. 1. *Displacement of dissolved acetylene by bubbling. Samples (3 ml) of water were incubated under 0.1 atm of C_2H_2 in stoppered serum bottles at 20°C for 30 min. Amounts of dissolved C_2H_2 were determined after bubbling N_2 through the water, at a rate of 48 ml/min, for 0 to 5 min.*

rupted treatment with acetylene (Fig. 2c) when rates were more than four times higher than in the control samples (treatment C, Fig. 2c).

A similar enhancement of acetylene-reducing activity occurs when the alga is exposed alternately to 10% acetylene in air and to ambient atmosphere (treatment B, Fig. 2d), with an almost fivefold increase of enzyme activity after a 4-h pretreatment with acetylene. When exposure to ambient atmosphere is further followed by bubbling N_2 gas through the cell suspension for 5 min before the nitrogenase assay, the rates of acetylene reduction are considerably lowered as compared with treatment B, though they are still 15 to 85% higher than those measured in the control samples (Fig. 2d).

Ethylene production was not detected when dead (boiled) cells were subjected to treatments A and B (Fig. 2c and d). No appreciable difference in the pattern of enhancement of acetylene reduction was noticed when samples subjected to treatment B were sealed with unused rubber stoppers before the acetylene-ethylene assay.

Experiments with *Anabaenopsis circularis* have, on the whole, confirmed the characteristic response to pretreatment with acetylene shown by *Anabaena cylindrica*.

Effect of preincubation under acetylene on acetylene-reducing activity in *Azotobacter vinelandii*. Treatments A and B have brought about a change in the rate of acetylene reduction by *Azotobacter vinelandii* similar to that observed with blue-green algae. Continuous incubation under 0.1 atm of acetylene resulted in a peak activity after 3 h of preincubation with

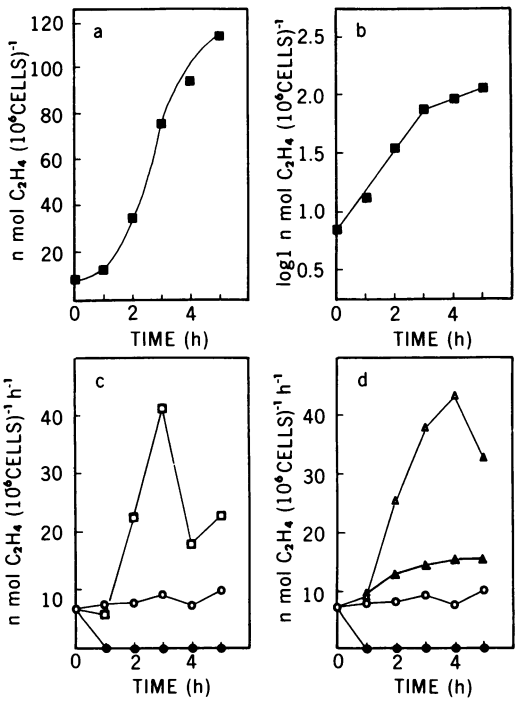

FIG. 2. *Effect of preincubation under acetylene (10% C_2H_2 in air) on the rate of acetylene reduction by Anabaena cylindrica. (a) Time course of C_2H_4 production during continuous incubation under C_2H_2; (b) logarithmic plot of the cumulative C_2H_4 production; (c) changes in the rate of C_2H_4 production during continuous incubation under C_2H_2 (□), compared with the untreated control samples (○) and treated boiled samples (●); (d) rates of C_2H_4 production after alternate incubation under C_2H_2 and ambient atmosphere, respectively (△), and when the latter was followed by bubbling N_2 through suspensions (▲), compared with the untreated control (○) and treated boiled samples (●).*

acetylene (Fig. 3a and b) when a 10-fold increase of enzyme activity was recorded. After a sharp decline, acetylene-reducing activity was partially recovered after 9 h of continued incubation with acetylene before it fell again almost to the initial value. Though there was no appreciable growth during continuous incubation with acetylene, new enzyme synthesis probably occurred and could be responsible for the observed fluctuations of enzyme activity. A similar, though less pronounced fluctuation was noticed during treatment B (alternating 0.1 atm of C_2H_2 with ambient atmosphere), which resulted after 12 h in rates of acetylene reduction three times higher than those in the control samples (Fig. 3b). When treatment B was modified to include a 10-min period of evacuation after a 20-min exposure of cell suspension to ambient atmos-

phere, the enhancement of acetylene-reducing activity, though slightly lowered, was still considerable (Fig. 3b).

Enhancement of acetylene-reducing activity in *R. rubrum*. In experiments with *R. rubrum* an anaerobic gas mixture, 1% CO_2 in N_2 (vol/vol), was provided to replace air. When cell suspensions were alternately exposed to 10% acetylene in the anaerobic gas mixture and to the anaerobic gas mixture, respectively (treatment B), a very marked increase of the acetylene-reducing activity was observed (Fig. 4).

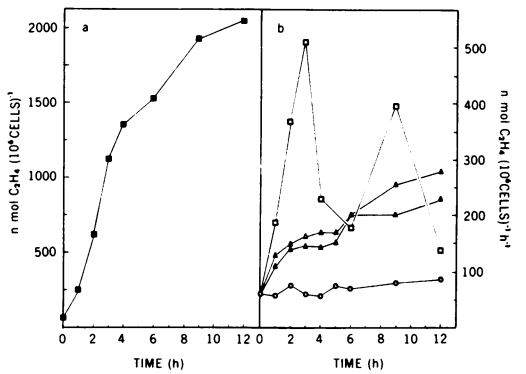

FIG. 3. *Effect of preincubation under acetylene (10% C_2H_2 in air) on the rate of acetylene reduction by Azotobacter vinelandii. (a) Time course of C_2H_4 production during continuous incubation under C_2H_2; (b) changes in the rate of C_2H_4 production during continuous incubation under C_2H_2 (□), after alternate incubation under C_2H_2 and ambient atmosphere, respectively (△), and when a similar treatment was supplemented with evacuation before the acetylene-ethylene assay (▲), compared with untreated control samples* (○).

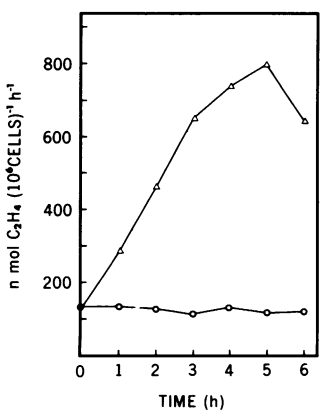

FIG. 4. *Effect of preincubation under acetylene (10% C_2H_2–1% CO_2–89% N_2) on the rate of ethylene production by R. rubrum, after alternate incubation with and without C_2H_2 (△), compared with untreated control samples* (○).

Maximum rates of acetylene reduction were measured after 5 h of preincubation with acetylene when the amounts of ethylene formed were 40 times higher than those produced by the control samples. No enhancement was observed, however, when cells were incubated continuously under the acetylene-containing gas phase (treatment A).

Effect of partial pressure of acetylene on the rate of acetylene reduction by *Anabaena cylindrica*. To test the effect of increased partial pressure (substrate concentration) on the rate of acetylene reduction, cell suspensions of *Anabaena cylindrica* were incubated under a gas phase containing acetylene at partial pressures of 0.1 and 0.2 atm of acetylene in air, respectively. Treatments were otherwise similar to those applied in the previous experiments.

It is clear from Fig. 5a to d that the responses of the alga to treatments with C_2H_2 of 0.1 and

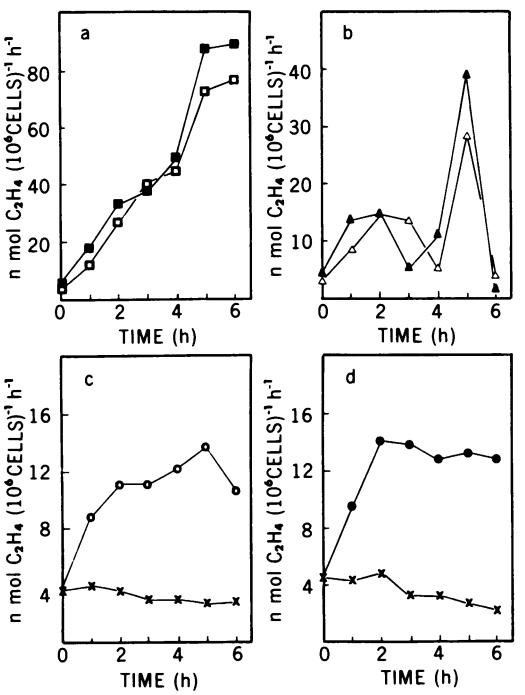

FIG. 5. *Effect of partial pressure of acetylene on the rate of acetylene reduction by Anabaena cylindrica. (a) Time course of C_2H_4 production during continuous incubation under 0.1* (□) *and 0.2* (■) *atm of C_2H_2; (b) changes in the rate of C_2H_4 production during continuous incubation under 0.1* (△) *and 0.2* (▲) *atm of C_2H_2; (c) rates of C_2H_4 production after alternate incubation under 0.1 atm of C_2H_2 and ambient atmosphere, respectively* (○), *compared with untreated control samples* (×); *(d) as in (c), but incubation under 0.2 atm of C_2H_2* (●).

0.2 atm were, on the whole, similar. The magnitude of enhancement of acetylene-reducing activity was scarcely, if at all, increased when the alga was incubated under 0.2 atm of acetylene.

Effect of preincubation with acetylene on ^{15}N incorporation in *Anabaena cylindrica*. In this series of experiments, N_2-fixing activity was assayed by the isotope method after preincubation of algal suspensions under 0.1 atm of acetylene. Replicate samples were subjected to treatments B (alternating 10% C_2H_2 in air with ambient atmosphere) and C (control) as described earlier. At hourly intervals triplicate suspension samples were exposed to a gas phase containing 10% $^{15}N_2$ (95-atom % enrichment), 1% CO_2, and the rest argon (vol/vol), and incubated at 20°C with illumination (1,500 lx) and continuous agitation for 30 min. The reaction was terminated by injection of mercuric sulfate into the cell suspension, and this was followed by Kjeldahl digestion, distillation, and ^{15}N analysis. Parallel series were assayed for acetylene reduction to allow direct comparison between the effect of preincubation with acetylene upon $^{15}N_2$ fixation and ethylene production by the algal nitrogenase.

Figure 6a shows the familiar pattern of enhancement of acetylene reduction induced by pretreatment with acetylene. Figure 6b illustrates the relative inhibition of ^{15}N incorporation after preincubation with acetylene. Maximum depression of N_2 fixation coincided with maximum enhancement of acetylene reduction after 3 h of preincubation with acetylene. When acetylene was completely removed, by bubbling N_2 gas through the suspensions for 5 min (Fig. 1), after treatment B and before exposure of the alga to $^{15}N_2$, the inhibitory effect, though eased considerably, had not been fully relieved.

Effect of preincubation with acetylene on photosynthesis in *Anabaena cylindrica*. Next the effect of preincubation under 0.1 atm of acetylene on the rate of photosynthetic $^{14}CO_2$ fixation in *Anabaena cylindrica* was investigated. Triplicate suspension samples, previously subjected to treatments A, B, and C, were exposed at hourly intervals to $^{14}CO_2$ in light (1,500 lx) at 20°C with continuous agitation for 30 min. The reaction was terminated by transfer of the suspension samples into a darkened icebox. Triplicate suspension samples were filtered and the algal material was collected on filter membranes for radioactivity assay.

Long-term treatment with acetylene led to a marked reduction in the rate of $^{14}CO_2$ incorporation in *Anabaena cylindrica*, especially when preincubation under 0.1 atm of acetylene alternated with periods of exposure to ambient atmosphere (treatment B). Maximum depression

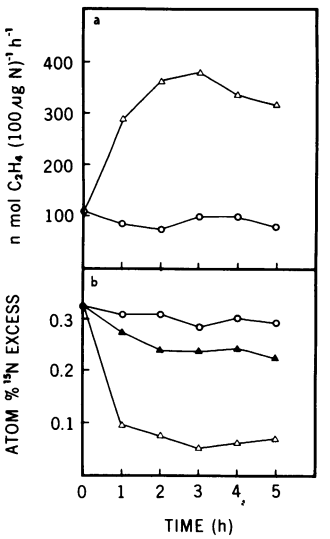

FIG. 6. *Effect of preincubation under acetylene (10% in air) on $^{15}N_2$ fixation by Anabaena cylindrica. (a) Changes in the rate of C_2H_4 production after alternate incubation under C_2H_2 and ambient atmosphere, respectively* (△), *compared with untreated control samples* (○); *(b) changes in the rate of ^{15}N incorporation after alternate incubation under C_2H_2 and ambient atmosphere, respectively* (△), *and when similar treatment was supplemented with bubbling N_2 before exposure to $^{15}N_2$* (▲), *compared with untreated control samples* (○).

(32% after treatment A and 62% after treatment B) was recorded after 3 h of preincubation with acetylene (Fig. 7). This coincided with maximum enhancement of acetylene reduction and maximum depression of N_2 fixation in the alga.

The nature of inhibition by acetylene of $^{14}CO_2$ fixation was examined in another experiment for which the alga was separately grown on different sources of inorganic nitrogen (N_2, nitrate, and ammonium-nitrogen) and then exposed to $^{14}CO_2$ at two selected wavelengths of light, 625 and 675 nm, for 30 min. Table 2 shows that ^{14}C assimilation was depressed to a greater extent in alga grown under N_2-fixing conditions and exposed to 675 nm of light than in alga grown on nitrate or exposed to light of 625-nm wavelength. No inhibition was observed in alga grown on ammonium-nitrogen.

DISCUSSION

Acetylene is a noncompetitive inhibitor of nitrogen fixation and prevents ammonia synthesis as long as it remains in the medium at a concentration sufficient to saturate nitrogenase. Such a condition leads to nitrogen depletion in cells, and may in turn induce derepression and new enzyme synthesis (16). The effect of long-term

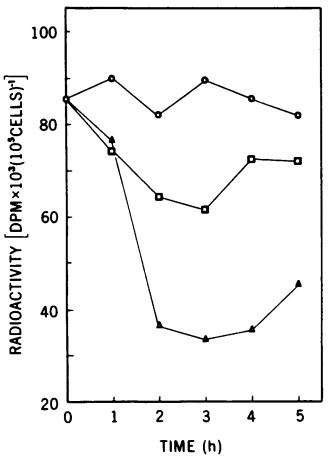

FIG. 7. *Effect of preincubation under acetylene (10% in air) on photosynthetic $^{14}CO_2$ fixation in Anabaena cylindrica. Suspension samples (3 ml) were supplied with 2 μCi of ^{14}C plus 10 μmol of $NaHCO_3$ and incubated in light (1,500 lx) at 20°C for 30 min. Changes in the rate of ^{14}C incorporation after continuous incubation with C_2H_2 (□), after alternate incubation under C_2H_2 and ambient atmosphere, respectively (△), and in the untreated samples* (○).

TABLE 2. *Effect of C_2H_2 on $^{14}CO_2$ fixation in Anabaena cylindrica grown on different sources of nitrogen and exposed to selected wavelengths of light*[a]

N source	Wavelength (nm)	Gas phase	dpm/(100 μW/cm²)/10⁵ cells per h
N_2	625	Air	3,800
		0.1 atm of C_2H_2	2,700 (29%)[b]
	675	Air	1,900
		0.1 atm of C_2H_2	460 (76%)
NO_3^-	625	Air	2,730
		0.1 atm of C_2H_2	2,030 (25%)
	675	Air	3,090
		0.1 atm of C_2H_2	1,840 (40%)

[a] Preincubation with and without C_2H_2 in light (1,500 lx) at 20°C for 60 min was followed by exposure of alga to $^{14}CO_2$ (2 μCi of ^{14}C and 10 μmol of $NaHCO_3$ per 3-ml suspension) for 30 min.

[b] Percent inhibition of CO_2 fixation is indicated in parentheses.

incubation under acetylene was considered to be one of the factors causing the rate of acetylene reduction to increase with time of incubation (9). In *Anabaena cylindrica* (as in other blue-green algae), nitrogen starvation leads to the utilization of usually ample cellular reserves of organic nitrogen before any appreciable increase of nitrogenase activity is observed (5). It appears, therefore, that other factors may also be involved.

Prolonged incubation under acetylene and the accompanying high rates of ethylene production constitute a considerable wastage of valuable resources on a physiologically useless reaction. Energy and reducing power generated in the photochemical reaction or at the expense of organic nutrients are diverted from metabolic and biosynthetic routes and used for the formation of a non-metabolizable product. It is obvious that such wasteful processes will in turn affect the supply of adenosine 5'-triphosphate and reductant for the nitrogenase-catalyzed reactions. Hence, sooner or later, a decline in the rate of acetylene reduction appears unavoidable.

It is not clear whether the inhibition by acetylene of CO_2 fixation in *Anabaena cylindrica* corresponds to a direct effect on the photosynthetic process or is the indirect consequence of nitrogen depletion. It is possible that acetylene reduction and CO_2 fixation compete for reductant. Though the time course of depression of ^{14}C incorporation tallies reasonably well with the time course of enhancement of acetylene reduction, the observation that no inhibition of $^{14}CO_2$ fixation occurred in alga grown on ammonium-nitrogen seems to suggest an indirect effect of acetylene on photosynthetic CO_2 fixation. The more marked inhibitory effect of acetylene on rate of CO_2 fixation when the alga was exposed to light of 675-nm wavelength may be related to the specific stimulation of nitrogenase activity in light selectively absorbed by chlorophyll *a* (7).

The inverse relationship between the rates of acetylene reduction and ^{15}N incorporation and the observation that inhibition by acetylene of $^{15}N_2$ fixation in *Anabaena cylindrica* persists (at least for a while) even after complete removal of acetylene from the suspending medium indicate that preincubation with acetylene affects the rate of subsequent N_2 fixation. The present study provides no information as to whether this is due to a conformational change on the enzyme molecule or is caused simply by a "memory" effect at the active site of substrate complexation and reduction. Although it is still unresolved whether there are separate catalytic sites on nitrogenase for different substrates or whether they all react at a common site, biochemical evidence suggests that significant differences may exist between the modes of complex formation between nitrogenase and any one substrate (2, 9, 12).

We may conclude that though the effects of prolonged incubation with acetylene upon nitrogen-fixing organisms are complex, they seem to arise primarily through the inhibition by acetylene of N_2 fixation and are partly due to the wasteful use of adenosine 5'-triphosphate and reductant in the acetylene reduction reaction. It is clear that the marked enhancement of acet-

ylene-reducing activity during long-term exposure to acetylene may yield gross overestimates of actual N_2 fixation in the field, where long periods of experimental incubation are most frequently applied. The standardization of experimental conditions and short-term assays are therefore an absolute necessity in order to obtain reliable information regarding the nitrogen-fixing potential of microorganisms with the acetylene-ethylene assay. This should preferably be performed in the absence of an alternative substrate and include a brief period of preincubation with acetylene, adequate to adapt and saturate the enzyme with substrate but insufficient to create conditions of nitrogen depletion.

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